

### Lysogenic *Klebsiella pneumoniae* Strains of Capsular Serotype 2(B)

Lysogenic strains of *Klebsiella pneumoniae* (*Aerobacter aerogenes*) of capsular serotypes 1(A) and 2(B) have been reported by PARK<sup>1</sup> and CIUCA et al.<sup>2</sup> These lysogenic strains released phages active on other strains of *K. pneumoniae*. The present communication reports the finding of four other lysogenic strains, of capsular serotype 2(B).

Stab inocula were made from overnight nutrient broth cultures of *K. pneumoniae* strains into nutrient agar plates bearing a soft agar overlay seeded with the prospective indicator strain. This method is similar to that employed by PAPAVALSILIOU<sup>3</sup> to detect lysogeny in *Bacterium anitratum* strains. *K. pneumoniae* strains which gave zones of inhibition on the plates were tested further. Aged nutrient broth cultures and broth cultures of ultra-violet irradiated bacteria were centrifuged and the supernatants examined for the presence of phage. These supernatants were sterilized by shaking with chloroform or by treatment with streptomycin. In the latter case the tests for phage were made on a plate seeded with a streptomycin-resistant mutant of the bacterial indicator strain.

Type 2(B) strains B7380, 2.2, 2.5, and 2.12 gave turbid zones of inhibition when tested against a type 2 strain obtained from Dr. B. H. PARK. Strains 2.5 and 2.12 are derivatives of N.C.T.C. strains 7761 and 9620 respectively. PARK<sup>1</sup> reported his type 2 strain itself to be lysogenic, but

none of the 48 capsulate and non-capsulate *K. pneumoniae* strains in my collection was sensitive to the phage released by the PARK strain. Tests of culture supernatants and dilutions showed that strains B7380, 2.2 and 2.5 are indeed lysogenic, discrete phage plaques being obtained on lawns of PARK's type 2 strain.

*Résumé.* L'auteur décrit quatre souches de *Klebsiella pneumoniae* du type capsulaire B lysogènes pour les bactériophages actifs pour une autre souche du même type.

C. H. CLARKE<sup>4</sup>

*Institute of Animal Genetics, West Mains Road, Edinburgh (Scotland), August 22, 1963.*

<sup>1</sup> B. H. PARK, *Virology* 2, 711 (1956).

<sup>2</sup> M. CIUCA, S. STAMATESCO-EUSTATZIOU, C. BARBER, M. VOINEA, and G. TULPAN, *Arch. Roum. Pathol. exp. Microbiol.* 18, 347 (1959).

<sup>3</sup> J. PAPAVALSILIOU, *J. Bacteriol.* 80, 138 (1960).

<sup>4</sup> I am grateful to Professors D. G. CATCHESIDE and J. P. DUGUID, Drs. B. H. PARK, I. ØRSKOV, J. PAPAVALSILIOU and Y. HAMON for encouragement, information and bacterial strains. This work was begun at the Microbiology Department, University of Birmingham, while the author held an award from the Agricultural Research Council.

### Isolation of

#### 2-Acetyl-2-decarboxamidotetracycline from Cultures of *Streptomyces psammoticus*

VIRGILIO and HENGELLER described *Streptomyces psammoticus* as a new species of streptomycetes capable of producing only tetracycline, even in the presence of considerable quantities of chloro-ions in the fermentation medium<sup>1</sup>. On the basis of the analytical methods which they employed, VIRGILIO and HENGELLER concluded that neither chlorotetracycline nor oxytetracycline were detectable in *S. psammoticus* cultures. On the other hand, HOCHSTEIN et al.<sup>2</sup> and MILLER and HOCHSTEIN<sup>3</sup> found that strains of streptomycetes which produce either 7-chlorotetracycline or 5-oxytetracycline also synthesize 2-acetyl-2-decarboxamidotetracyclines. These authors pointed out that the capacity of *S. rimosus* and *S. aureofaciens* to synthesize such products is indicative of a biogenetic relationship between the tetracycline and the class of the 'indicator' antibiotics which are characterized by a considerably unsaturated chromophoric nucleus of naphthacene structure.

It seemed worthwhile to investigate whether also *Streptomyces psammoticus* cultures could synthesize small quantities of 2-acetyl-2-decarboxamidotetracycline together with tetracycline. Fermentations with *S. psammoticus* were carried out at 28°C in glass jars of 4 l capacity, stirred at 750 r.p.m. and aerated at 0.75 litre/litre of medium/min. The fermentation medium consisted of: corn steep 20 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 9 g; glucose 60 g; MgSO<sub>4</sub> · 7H<sub>2</sub>O 1 g; CaCO<sub>3</sub> 9 g; tap water 1 l; pH, before sterilization 6.8. After 96 h the concentration of the antibiotic produced was 2030 µg/ml.

The fermentation broth was acidified to pH 2.0, filtered, and extensively extracted with *n*-butanol after addition

of 15% w/v of sodium chloride. The combined extracts were evaporated under reduced pressure to a small volume, the precipitated sodium chloride removed by filtration and the tetracycline was reextracted in water by addition of petroleum ether to the butanol.

Reextraction of the tetracycline in butanol and addition of ethyl ether to the concentrated butanolic extract yielded a precipitate of crude tetracycline hydrochloride with a microbiological activity of 550 µg/mg. A 1.82 g sample of this crude product (corresponding to 1 g of pure tetracycline hydrochloride) was fractionated by countercurrent distribution. The fundamental procedure described by CRAIG was employed, using 10 ml per tube of each phase of a solvent system consisting of 0.01 *N* hydrochloric acid and *n*-butanol; 230 transfers were carried out. When the distribution was complete, a sample (1 ml of each phase) of every fifth tube was analysed by determining the extinction at 360 mµ after dilution with methanol 0.01 *N* in hydrochloric acid. The results are given in the Figure.

In addition to the main peak, which was at the 66th tube (*K* = 0.40) and which corresponds to tetracycline, a second small maximum was found at about the 109th tube (*K* = 0.9). Spectrophotometric analysis of the 100th to the 120th tubes showed maxima at 276 mµ and 360 mµ, whereas tetracycline gives maxima at 268 mµ and 360 mµ.

In order to isolate an amount of the substance *K* = 0.9 for identification purposes, the countercurrent distribu-

<sup>1</sup> A. VIRGILIO and C. HENGELLER, *Il Farmaco*, Ed. Sci. 15, 164 (1960).

<sup>2</sup> F. A. HOCHSTEIN, M. SCHACH VON WITTENAU, F. W. TANNER, and K. MURAI, *J. Amer. chem. Soc.* 82, 5934 (1960).

<sup>3</sup> M. W. MILLER and F. A. HOCHSTEIN, *J. org. Chem.* 27, 2525 (1962).

tion experiment was repeated five times, using each time 1.8 g of crude tetracycline hydrochloride. The contents of the 107th to 120th tubes were collected and extracted in butanol (4 extractions with equal volumes of the solvent). The butanol was washed with water, concentrated to 1–2 ml, and poured into petroleum ether. The amorphous precipitate was dissolved in 1 ml water and the pH of the solution was brought to 5.0 by the addition of 1.0 *N* sodium hydroxide. Crystallization of a yellow product occurred. The crystals were filtered and dried 4 h at 60°C *in vacuo*.

M.p. 178–185° (dec)

$[\alpha]_D = -125^\circ$  ( $C = 1.0$ , in methanol 0.5 *N* HCl)

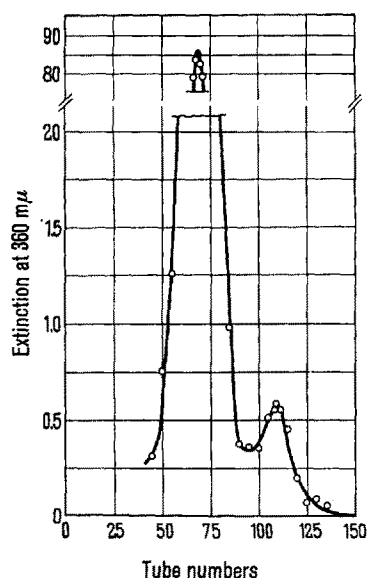
	C	H	N
Analysis Calculated for $C_{22}H_{40}NO_8 \cdot H_2O$	59.86	5.90	3.04
Found	59.90	6.20	3.01

UV-spectrum	$\lambda$ max	$E_{1\%}^{1\text{cm}}$
in methanol 0.01 <i>N</i> HCl	276	312
	359	300
in methanol 0.01 <i>N</i> NaOH	270	388
	382	367

IR-spectrum: Principal bands (in nujol):

3300, 1650, 1605, 1505, 1340, 1254, 1225, 1208, 1180, 1130, 1117, 1102, 1060, 1043, 1005, 963, 945, 920, 857, 839, 820  $\text{cm}^{-1}$ .

After boiling in 2 *N* HCl for 5 min, the product gave an ultra-violet and visible spectrum characteristic of an anhydroderivative, with maxima at 273 and 427  $\text{m}\mu$ .



Counter-current distribution of the crude tetracycline extracted from *S. psammoticus* cultures. Solvent system: butanol/0.01 *N* HCl; 230 transfers.

The microbiological activity was determined according to KERSEY's method, using *Klebsiella pneumoniae* P.C.I. 602<sup>4</sup> as test organism, and was found to be 4.62% of that of oxytetracycline.

From the characteristics given above and from a comparison with an authentic sample<sup>5</sup>, the product was identified as 2-acetyl-2-decarboxamido-tetracycline, as described by MILLER and HOCHSTEIN<sup>6</sup>.

The chromatographic behaviour of 2-acetyl-2-decarboxamidotetracycline in various solvents is given in the Table. (The *R<sub>f</sub>* values for tetracycline, oxytetracycline, and chlorotetracycline in the same solvent systems are also given.) The solvent system D appears to be useful for the chromatographic separation from the other common tetracyclines.

#### Chromatographic behaviour of 2-acetyl-2-decarboxamidotetracycline

	R <sub>f</sub> values in various solvent systems			
	A	B	C	D
Tetracycline	0.37	0.75	0.39	0.36
5-Oxytetracycline	0.37	0.67	0.18	0.50
7-Chlorotetracycline	0.65	0.80	0.64	0.75
2-Acetyl-2-decarboxamido-tetracycline	0.67	0.80	0.20	0.17

(A) Butanol saturated with water<sup>8</sup>, Whatman No. 1 paper, saturated with phosphate buffer pH 3.0. (B) Butanol, acetic acid, water (12:3:5), Whatman No. 1 paper, saturated with buffer pH 3.0. (C) Chloroform, nitromethane, pyridine (10:20:3)<sup>7</sup>, Whatman No. 4 paper, wet with McIlvaine buffer pH 3.5. (D) Ethyl acetate, benzene, pyridine (20:10:3), Whatman No. 4 paper, wet with McIlvaine buffer pH 3.5.

Bioautography on plates seeded with *Bacillus subtilis*.

**Riassunto.** Lo *Streptomyces psammoticus* produce in coltura sommersa oltre a preponderanti quantità di tetraciclina anche piccole quantità di 2-acetil-2-decarbrossamidotetraciclina. Sono riportati i dati cromatografici per distinguere qualitativamente la 2-acetil-2-decarbrossamidotetraciclina dalle più comuni tetracicline.

G. C. LANCINI and P. SENSI

Laboratori Ricerche Lepetit, Milano (Italy),  
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<sup>4</sup> R. C. KERSEY, J. Amer. pharm. Assoc. Sci. Ed. 39, 252 (1950).

<sup>5</sup> We express our thanks to Dr. MILLER (Pfizer), who kindly supplied us with a reference sample of 2-acetyl-2-decarboxamidotetracycline.

<sup>6</sup> N. BOHONOS, A. C. DORNBUSH, L. I. FELDMAN, J. H. MARTIN, E. PELCAK, and J. H. WILLIAMS, Antibiotics Annual 1953–54, 49.

<sup>7</sup> G. B. SELZER and W. W. WRIGHT, Antib. Chemother. 7, 292 (1957).